REMARKS

Please enter this Amendment. Applicants respectfully solicit an early favorable action on the merits.

Applicants acknowledge with appreciation the Examiner's entry of the corrected sequence listing and the computer readable form thereof filed February 25, 2004.

Applicants present an amended claim 1 in an effort to adapt the Examiner's constructive suggestions. The Examiner's constructive comments are acknowledged with appreciation. Amended claim 1 recites isolated polynucleotide sequence in the preamble and it is respectfully suggested that this addresses the rejection under 35 U.S.C. §101, and makes the reference to 'isolated' in claim 1 c) redundant whereby it is deleted in the amendment. Claim 1 d) recites poloynucleotide sequence in response to the Examiner's suggestion in the Office Action at page 2 bridging to page 3. Claim 1 d) is also amended to recite "an amino acid sequence comprising SEQ ID NO:1 having 6 additional amino acids..." in an effort to address the Examiner's comments in the Office Action at page 7. Amended claim 1 deletes paragraph e) withouth prejudice.

Applicants have canceled claim 40 and amended claim 9.

New claims 42 and 43 relate to a disclosed exemplary species, which is apparently available to those skilled in the art from recognized microorganism depositories, namely *Bacillus megaterium* IFO12108.

It is respectfully suggested that the amendments to claim 1 will address formality objections/rejections and the rejection under 35 U.S.C. §101 and thus reduce issues for further consideration. Applicants respectfully request the Examiner to approve this claim amendment.

It is respectfully suggested that presentation of new claims 42 and 43 does not present a new issue or necessitate a new search. New claims 42 and 43 refer to the microorganism species *Bacillus megaterium* IFO12108 and are supported by the written description in the original specification. The species is cited in the Office Action. These new claims are submitted so as to have claims responding to the comment in the Office Action, page 3, regarding claims 40 and 41.

Non-Elected Claims:

Non-elected claims may be canceled by Examiner's Amendment upon indication of allowable, elected subject matter.

Information Provided Pursuant to *In re Eynde*:

Applicants respectfully invite the Examiner's attention to the attached literature references that refer to and/or describe glucose dehydrogenase and *Bacillus megaterium*. The literature is submitted consistent with the principles of In re Eynde, 480 F.2d 1364 (CCPA 1973)[1] to support the enablement of claims reciting "enzyme in B) is glucose dehydrogenase derived from *Bacillus megaterium*" or comparable language. The literature enclosed herewith is:

1) T. Nagao et al., Cloning, nucleotide sequences, and enzymatic properties of glucose dehydrogenase isozymes from bacillus magaterium IAM 1030, J. Bacteriology,

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[1] "That statutory requirement [of Section 112] is fulfilled where one possessed of the knowledge had by one skilled in the art could use the invention given the specification disclosure without undue experimentation. A patent applicant may offer evidence, such as patents and publications, to show the knowledge possessed by those skilled in the art and thereby establish that a given specification disclosure is enabling. See, e.g., Martin v. Johnson, 59 CCPA , 454 F.2d 746, 172 USPQ 391 (1972). In such a situation, it is the knowledge possessed by those skilled in the art as of the filing date that is of relevance. See Tummers v. Kleimack, ...455 F.2d 566, 568, 172 USPQ 592, 593 (1972)."

174:5013-5020 (Aug. 1992) (Abstract from American Society for Microbiology web site);

2) DNA vector systems, Bacillus megaterium Expression System, two pages, from mobitec-germany web site;

3) Bacteria listing from ATCC web site to show exemplary deposits for Bacillus megatetium and also identifying representative literature citations (one page);

4) Extract from web site listing for enzymes EC 1.1.1.47, which is apparently assoicated with the text Enzyme Nomenclature, International Union of Biochemistry (1984 and/or subsequent editions), showing glucose 1-dehydrogenase has other names known to those skilled in the art, and identifying exemplary supporting literature references;

5) Extract from U.S. Department of Commerce web site materials describing glucose dehydrogenase and identifying a literature citation;

6) Extract from USB - Biochemicals' web site relating to glucose dehydrogenase (E.C. 1.1.1.47);

7) Extract from X-Zyme web site relating to D-glucose dehydrogenase from Bacillus megaterium identifying a literature citation; and

8) Yamamoto et al., Crystal Structure of Glucose Dehydrogenase from *Bacillus megaterim* IWG3 at 1.7 Å Resolution, J. Biochem., 129:303-312 (2001) from The Japanese Biochemical Society.

The enclosed information is readily available to any person skilled in the art since the literature was developed via a simple internet search.

It is respectfully requested that it be considered in connection with at least claims 9 (amended) and 41.

Traversing the Rejection of Claims 9, 11-14, 40 and 41.

Claims 9, 11-14, 40 and 41 were rejected under 35 U.S.C. §112 (¶1) as containing subject matter which allegedly was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time their application was filed, had possession of the claimed inventions.

Applicants traverse. Their specification as filed contained a written description of their inventions that reasonably conveyed to a person skilled in the art that the inventors had possession of the claimed inventions.

Claim 9 and claim 14 find support in the original specification throughout, including Examples 2 and 3 (page 64). It will be appreciated that a commericially available glucose dehydrogenase is disclosed, and such an enzyme is recited in claims 9 and 14.

The literature cited herewith that is available via simply internet search further substantiates the Applicants' position that a glucose dehydrogenase enzyme was available and that an exemplary microorganism(s) was also available. In re Eynde, *supra*. It is suggested this evidence suffices to rebut the hypothesis offered in the Office Action.

Furthermore, Claim 41 and new claims 42 and 43 are thought to be free of this rejection. Claims 42 and 43 are to a species of micororganism further identified by deposit.

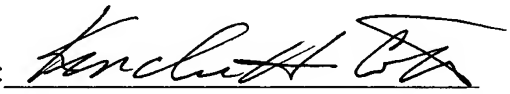
Traversing the Additional Rejection Under 35 U.S.C. 112(1):

Applicants respectfully submit the rejection of claims 1-9, 11-14 and 39-41 under 35 U.S.C. §112(¶1) should be reconsidered and withdrawn. The Examiner objected to claim 1, paragraph e), which is now canceled, whereby the rejection is moot. The cancelation is without prejudice to pursing such subject matter in a further application.

Conclusion:

Applicants respectfully submit they have endeavored to respond fully to the Office Action and their Amendment places their application in condition to receive a notice of allowance.

Respectfully submitted,
FITCH, EVEN, TABIN & FLANNERY

By: 

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J. Bacteriol., Aug 1992, 5013-5020, Vol 174, No. 15

Cloning, nucleotide sequences, and enzymatic properties of glucose dehydrogenase isozymes from Bacillus megaterium IAM1030

T Nagao, T Mitamura, XH Wang, S Negoro, T Yomo, I Urabe and H Okada
Department of Biotechnology, Faculty of Engineering, Osaka University, Japan.

Bacillus megaterium is known to have several genes that code for isozymes of glucose dehydrogenase. Two of them, gdhI and gdhII, were cloned from B. megaterium IAM1030 in our previous work (T. Mitamura, R. V. Evora, T. Nakai, Y. Makino, S. Negoro, I. Urabe, and H. Okada, J. Ferment. Bioeng. 70:363-369, 1990). In the present study, two new genes, gdhIII and gdhIV, were isolated from the same strain and their nucleotide sequences were identified. Each gene has an open reading frame of 783 bp available to encode a peptide of 261 amino acids. Thus, a total of four glucose dehydrogenase genes have been cloned from B. megaterium IAM1030. In addition, this strain does not seem to have other glucose dehydrogenase genes that can be distinguished from the four cloned genes so far examined by Southern hybridization analysis. The two newly cloned genes were expressed in Escherichia coli cells, and the products, GlcDH-III and GlcDH-IV, were purified and characterized and compared with the other isozymes, GlcDH-I and GlcDH- II, encoded by gdhI and gdhII, respectively. These isozymes showed different mobilities in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (GlcDH-I greater than GlcDH-III = GlcDH-IV greater than GlcDH-II), although they have the same number of amino acid residues. Double-immunodiffusion tests showed that GlcDH-I is immunologically different from the other isozymes and that GlcDH-III and GlcDH-IV are identical to one another but a little different from GlcDH-II. These glucose dehydrogenases were stabilized in the presence of 2 M NaCl. (ABSTRACT TRUNCATED AT 250 WORDS)

This article has been cited by other articles:

- van der Oost, J., Voorhorst, W. G. B., Kengen, S. W. M., Geerling, A. C. M., Wittenhorst, V., Gueguen, Y., de Vos, W. M. (2001). Genetic and biochemical characterization of a short-chain alcohol dehydrogenase from the hyperthermophilic archaeon Pyrococcus furiosus. *Eur J Biochem* 268: 3062-3068 [Abstract] [Full Text]
- Hanson, T. E., Tabita, F. R. (2001). A ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO)-like protein from Chlorobium tepidum that is involved with sulfur metabolism and the response to oxidative stress. *Proc. Natl. Acad. Sci. U. S. A.* 98: 4397-4402 [Abstract] [Full Text]
- Wada, M., Yoshizumi, A., Nakamori, S., Shimizu, S. (1999). Purification and Characterization of Monovalent Cation-Activated Levodione Reductase from Corynebacterium aquaticum M-13.

Appl. Environ. Microbiol. 65: 4399-4403 [Abstract] [Full Text]

Stable protein expression with high yield - suited **not only for industrial scale**

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DNA Vector Systems

Bacillus megaterium Expression System

Features:

- stable protein expression with high yield
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- polylinker downstream of promoter allows versatile cloning
- no indication of proteolytic instability even up to 5 hours after induction, since alkaline proteases (such as e.g. in *B. subtilis*) are not produced
- endotoxins are not found in the cell wall
- suited for industrial large scale protein production
- all ***B. subtilis*** vectors are compatible

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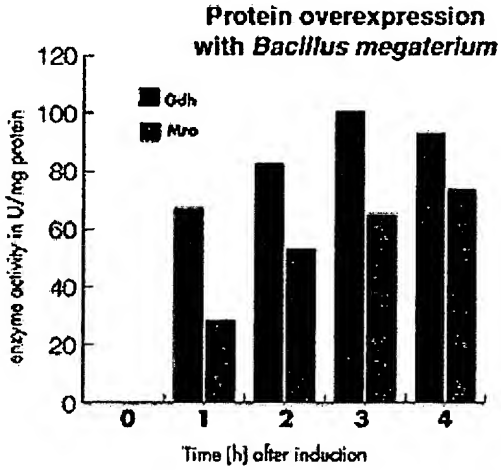
Our *Bacillus megaterium* kit offers an interesting **alternative to the standard host *E. coli*.** The kit comes with the *E. coli / Bacillus megaterium* shuttle vector pWH1520 and *B. megaterium* protoplasts ready for transformation. *B. megaterium* has proven to be an excellent host for the expression of non-homologous DNA. Over other bacilli strains it has the advantage, that none of the alkaline proteases are present. This fact enables cloning and expression of foreign proteins without degradation. In addition, there are no endotoxins found in the cell wall. Protein yields are exceptionally good, also if inexpensive substrates are used. Using the tightly regulated xylose operon the **genes were 130- to 350-fold induced without proteolysis.** A polylinker downstream of the promoter allows versatile cloning in pWH1520. All *B. subtilis* vectors are compatible with *B. megaterium* as well.
The *B. megaterium* system offers unique possibilities for the **industrial production** of proteins. In a diagnostic test for AIDS e.g., the HIV coat protein is commercially produced by *B. megaterium* (Ginsburgh *et al.*, 1989).

Examples:

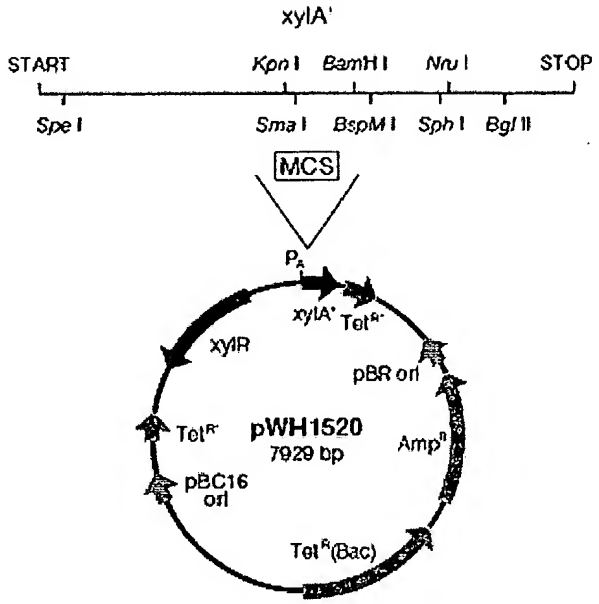
Proteins successfully over-produced with this system are:

- catabolite control protein (ccpA)
- xylose repressor (XylR)
- trehalose repressor (TreR)
- heat shock protein (HPr) from PTS (phosphotransferase sugar transport system)
- mutarotase (Mro)
- glucose dehydrogenase (Gdh)
- beta-galactosidase
- human single-chain urokinase-like plasminogen activator (rscuPA)
- cellulase



Strategy for high-efficient selection of recombinant clones.

Vector Map:



ORDER INFORMATION, SHIPPING & STORAGE:

order#	description	amount
BMEG01*	Bacillus megaterium kit:	
	• pWH1520; lyophilized DNA (*E. coli/B. megaterium* shuttle vector)	5 µg
	• *B. megaterium* protoplasts ready for transformation (strain WH320) Material is sufficient for 4 transformations plus control experiment; transformation protocol is supplied	5 x 500 µl

shipped on dry ice; DNA store at 4°C, protoplasts at -70°C, *not available in Europe

BMEG02	*B. megaterium* protoplasts	5 x 500 µl

shipped on dry ice; store at -70°C

BMEG03*	pWH1520*; lyophilized DNA	5 µg

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Bacteria

ATCC Number	Description	Designation	View
10778	*Bacillus megaterium* de Bary	NCIB 8508 [NRRL B-938]	☐
11478	*Bacillus megaterium* de Bary	Hershey PR3E	☐
11561	*Bacillus megaterium* de Bary	C9	☐
11561a	*Bacillus megaterium* de Bary	C9(M1)	☐
11561b	*Bacillus megaterium* de Bary	C9(M2)	☐
11561c	*Bacillus megaterium* de Bary	C9(M3)	☐
11561d	*Bacillus megaterium* de Bary	C9(M4)	☐
11561e	*Bacillus megaterium* de Bary	C9(M5)	☐
11562	*Bacillus megaterium* de Bary	899	☐
12872	*Bacillus megaterium* de Bary	QMRDC B1551	☐

Broberg P, Welsch M, Smith, Glucose dehydrogenase of Bacillus megaterium KM. Coupling of the cytoplasmic enzyme with membrane-bound cytochromes, Biochim Biophys Acta. 1969 Feb 25;172(2):205-15.

Baik SH, Ide T, Yoshida H, Kagami O, Harayama S., Significantly enhanced stability of glucose dehydrogenase by directed evolution., Appl Microbiol Biotechnol. 2003 May;61(4):329-35. Epub 2003 Mar 05.

T Nagao, T Mitamura, XH Wang, S Negoro, T Yomo, Urabe and H Okada, Cloning, nucleotide sequences, and enzymatic properties of glucose dehydrogenase isozymes from Bacillus megaterium IAM1030, J. Bacteriol., Vol 174(15):5013-5020 (Aug. 1992)

Pal, PG; Jany, KD; Saenger, W, Crystallization of and X-ray investigations on glucose dehydrogenase from Bacillus megaterium, Eur J Biochem 167, 123- 124, 1987.

Extract from web site

EC 1.1.1.47

Common name: glucose 1-dehydrogenase

Reaction: β-D-glucose + $NAD(P)^+$ = D-glucono-1,5-lactone + NAD(P)H + H^+

Other name(s): D-glucose dehydrogenase (NAD(P)); hexose phosphate dehydrogenase

Systematic name: β-D-glucose:$NAD(P)^+$ 1-oxidoreductase

Comments: Also oxidizes D-xylose.

Links to other databases: BRENDA, EXPASY, GTD, KEGG, ERGO, CAS registry number: 9028-53-9

References:

1. Banauch, D., Brummer, W., Ebeling, W., Metz, H., Rindfrey, H., Lang, H., Leybold, K. and Rick, W. A glucose dehydrogenase for the determination of glucose concentrations in body fluids. *Z. Klin. Chem. Klin. Biochem.* 13 (1975) 101-107. [Medline UI: 76057414]

2. Brink, N.G. Beef liver glucose dehydrogenase. 1. Purification and properties. *Acta Chem. Scand.* 7 (1953) 1081-1089.

3. Pauly, H.E. and Pfleiderer, G. D-Glucose dehydrogenase from *Bacillus megaterium* M 1286: purification, properties and structure. *Hoppe-Seyler's Z. Physiol. Chem.* 356 (1976) 1613-1623.

4. Strecker, H.J. and Korkes, S. Glucose dehydrogenase. *J. Biol. Chem.* 196 (1952) 769-784.

5. Thompson, R.E. and Carper, W.R. Glucose dehydrogenase from pig liver. I. Isolation and purification. *Biochim. Biophys. Acta* 198 (1970) 397-406. [Medline UI: 70151097]

Macromolecule -- dehydrogenase, glucose (M0OJ)

Molecule Name : dehydrogenase, glucose
Alias Names :
Genus: Bacillus
Species: megaterium M1286
Total Molecular Weight: 120000 **Total No. Subunits:** 4

Total Number of Crystal Entries: 1

1. Crystal C130: A=150.3, B=104.2, C=67.4, alpha=90, beta=90, gamma=90

Smith LD, Budgen N, Bungard SJ, Danson MJ, Hough DW, Purification and characterization of glucose dehydrogenase from the thermoacidophilic archaebacterium Thermoplasma acidophilum, Biochem. J. (1989) 261 (973–977) (Printed in Great Britain)

Glucose dehydrogenase was purified to homogeneity from the thermoacidophilic archaebacterium Thermoplasma acidophilum. The enzyme is a tetramer of polypeptide chain Mr 38,000 +/- 3000, it is catalytically active with both NAD+ and NADP+ cofactors, and it is thermostable and remarkably resistant to a variety of organic solvents. The amino acid composition was determined and compared with those of the glucose dehydrogenases from the archaebacterium Sulfolobus solfataricus and the eubacteria Bacillus subtilis and Bacillus megaterium. The N-terminal amino acid sequence of the Thermoplasma acidophilum enzyme was determined to be: (S/T)-E-Q-K-A-I-V-T-D-A-P-K-G-G-V-K-Y-T-T-I-D-M-P-E.

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	5000 units	$338.00
	10 ku	$568.00

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Form: Lyophilized powder

Unit Definition: One unit oxidizes 1 µmol of D-Glucose/min at 37°C.

Storage: 4°C

CAS #: 9028-53-9

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D-Glucose dehydrogenase

From Bacillus megaterium

D-Glucose: NADP 1-oxidoreductase
EC 1.1.1.47

Cat No. E-05-02 Version 2003

Store at -20°C

Product description

Application	Diagnostics and Cofactor regeneration of NADPH in reductive processes
Substrate specificity and Km	D-Glucose and D-Xylose KM VALUE [mM]: 4.6 beta-D-glucose, 1.9 NADP
pH optimum	8.0
M_r and structure	Tetramer alpha4, 4 * 30000, SDS-PAGE
Activity	200 U/mg át 30 °C
Formulation	In glycerol
Stability	Stable at -20 °C

REACTION	beta-D-glucose + NADP+ = D-glucono-1,5-lactone + NADPH
COMMENTARY	at pH 9.0 tetrameric enzyme dissociates into inactive subunits, inactivation proceeds by two distinct phases, 50% inactivation at the rapid first phase, 50% at slow second phase
Unit definition	1 U corresponds to the amount of enzyme which will oxidizes 1 µmol b-D-glucose to D-glucono-d-lactone per minute at pH 8.0 and 37°C
References	1. Yamamoto, K.; Kurisi, G.; Kusunoki, M.; Tabata, S.; Urabe, I.; Osaki, S., Crystal structure of glucose dehydrogenase from Bacillus megaterium IWG3 at 1.7 A resolution, J. Biochem.,129, 303-312, 2001

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[J. Biochem. Vol. 129, pp. 303-312 (2001), Regular paper; © 2001 by The Japanese Biochemical Society]

Crystal Structure of Glucose Dehydrogenase from *Bacillus megaterium* IWG3 at 1.7 Å Resolution[1]

Keizo Yamamoto,[*,2] Genji Kurisu,[†] Masami Kusunoki,[†] Shiro Tabata,[*] Itaru Urabe,[‡] and Shigeyoshi Osaki[*]

[*]Department of Chemistry, Nara Medical University, Shijo, Kashihara, Nara 634-8521; [†]Institute for Protein Research, Osaka University, Suita, Osaka 565-0871; and [‡]Department of Biotechnology, Graduate School of Engineering, Osaka University, Suita, Osaka 565-0871

Received October 16, 2000; accepted November 30, 2000

The crystal structure of glucose dehydrogenase (GlcDH) from *Bacillus megaterium* IWG3 has been determined to an *R*-factor of 17.9% at 1.7 Å resolution. The enzyme consists of four identical subunits, which are similar to those of other short-chain reductases/dehydrogenases (SDRs) in their overall folding and subunit architecture, although cofactor binding sites and subunit interactions differ. Whereas a pair of basic residues is well conserved among $NADP^+$-preferring SDRs, only Arg39 was found around the adenine ribose moiety of GlcDH. This suggests that one basic amino acid is enough to determine the coenzyme specificity. The four subunits are interrelated by three mutually perpendicular diad axes (P, Q, and R). While subunit interactions through the P-axis for GlcDH are not so different from those of the other SDRs, those through the Q-axis differ significantly. GlcDH was found to have weaker hydrophobic interactions in the Q-interface. Moreover, GlcDH lacks the salt bridge that stabilizes the subunit interaction in the Q-interface in the other SDRs. Hydrogen bonds between Q-axis related subunits are also less common than in the other SDRs. The GlcDH tetramer dissociates into inactive monomers at pH 9.0, which can be attributed mainly to the weakness of the Q-axis interface.

Key words: crystal structure, dissociation-association, glucose dehydrogenase, short-chain dehydrogenases/reductases, subunit interaction.

[email to author] • [Related articles in JB] • [Home page]

Glucose dehydrogenase (GlcDH; EC 1.1.1.47) catalyzes the oxidation of D-glucose to D-glucono-δ-lactone in the presence of coenzyme NAD^+ or $NADP^+$. $NAD(P)^+$

dependent GlcDH is produced by *Bacillus* species during endospore formation (1-3) and has been suggested to play a role in spore germination (4-6). The purified enzymes from *B. cereus* (1, 7), *B. megaterium* M1286 (2), *B. megaterium* IAM1030 (8), and *B. subtilis* (4) have been characterized, and the GlcDH genes from *B. subtilis* (9) and from different strains of *B. megaterium*, M1286 (10), IWG3 (11), and IAM1030 (12-14), have been cloned and the corresponding expressed enzymes characterized. Amino acid sequence alignment revealed that GlcDHs from these *Bacillus* species have more than 80% homology (14).

Glucose dehydrogenase from *B. megaterium* is a tetrameric enzyme (M_r 112,800) with four identical subunits (8, 11, 12, 15). GlcDH belongs to the family of short-chain dehydrogenases/reductases (SDRs) (16). More than 1,000 SDR DNA sequences have been registered in the sequence database (17), and more than 50 of these enzymes have been characterized (16). The active form of SDR enzymes is either a tetramer or a dimer, and each subunit typically consists of about 250 amino acid residues (16).

The three-dimensional structures of more than 10 members of the SDR family have been determined in the last decade (18-37). Despite their low sequence identities (no more than 30%), the three-dimensional structures of these enzymes show striking similarities in overall folding and intersubunit contacts. Accordingly, the overall structure of GlcDH is inferred to resemble those of the tetrameric SDRs of known structure. To date, however, only GlcDH shows a reversible dissociation-association of subunits under moderate conditions. The enzyme is inactivated in alkaline solution because of the dissociation of the tetramer into inactive monomers, which can reversibly associate into the fully active tetramer when the pH is lowered to 6.5 (38-40). The addition of 3 M NaCl prevents the alkaline dissociation, thereby stabilizing the tetramer structure (39).

To understand this remarkable feature that distinguishes GlcDH from other SDRs, information on the three-dimensional structure is indispensable. Here we report the refined crystal structure of glucose dehydrogenase from *B. megaterium* IWG3 complexed with NAD^+ at 1.7 Å resolution, showing detailed subunit interactions and a comparison of the intersubunit interactions with those of other tetrameric SDR enzymes. The amino acid residues that determine coenzyme specificities are also discussed.

MATERIALS AND METHODS

Crystallization and Data Collection

Crystallization and data collection were described previously (41). Briefly, crystals of GlcDH in complex with NAD^+ were obtained by the hanging-drop vapor diffusion method with the micro-seeding technique (42) using PEG2000 as a precipitant. Rod-shaped crystals grew to a maximum size of 0.25 x 0.25 x 3.0 mm in 2 weeks.

A data set for structure analysis was collected on synchrotron radiation at the beamline BL-18B of the Photon Factory operated at 2.5 GeV at the High Energy Accelerator Research Organization, Japan. Reflections were recorded on 400 mm x 800 mm imaging plates mounted on a screenless Weissenberg camera for macromolecular crystals (43) with a cylindrical cassette of 430 mm radius at 290 K. The latent images were digitized on a Rigaku SOR-DS48 scanner. The X-ray images were processed and scaled with the programs DENZO and SCALEPACK (44).

Molecular Replacement

The crystal structure was solved by molecular replacement with the program AMoRe (45). Coordinates of 3α,20β-hydroxysteroid dehydrogenase (3α-HSDH, PDB code 2HSD) having 35.0% sequence identity with GlcDH was used as a search model. Since subunit architecture in the asymmetric unit, dimer or tetramer, was unknown for GlcDH, a dimer unit formed by chains A and C of 3α-HSDH was used. All amino acid residues except for glycine in 3α-HSDH were replaced by alanine for the calculation. Since the cross-rotation functions did not give a definite single solution, each of the six most probable solutions with higher correlation coefficients was subjected to subsequent translation function calculations. The solution giving the highest correlation coefficient of translation function was selected to further search for translational solution of a second dimer unit in repetition. Crystal packing was analyzed for each dimer using the program QUANTA (Molecular Simulations, Burlington MA, USA) to select the final four subunits. The best solution gave a correlation coefficient of 29.7% and an *R*-factor of 51.0% after rigid-body refinement using the program AMoRe. Two kinds of tetramers in the unit cell having different orientations were found as shown in Fig. 1.

Model Building and Structure Refinement

Structure refinement was carried out initially with the program XPLOR, version 3.84 (46), and at the later stages with the program CNS, version 0.9 (47). The model obtained by the molecular replacement calculation was used as the initial model. Improvement of the model was monitored with a free *R*-value calculated from 5% of randomly selected reflections, which were excluded from the refinement procedure. The asymmetric unit is represented by chains A, B, E, and F. In the initial stage of refinement, non-crystallographic symmetry restraints were applied separately between chains A and B and between chains E and F. Each chain pair belonged to the same tetramer. Alanine side chains of the model were replaced with the corresponding side chain of GlcDH in several steps during refinement, giving priority to regions of higher sequence identity in order to avoid model biases. Iterative refinement cycles by XPLOR were performed until the *R*-value dropped below 30% (R_{free} = 36.9%). Subsequently the molecular model was manually rebuilt in the maps with coefficients of sigma weighted $2F_o - F_c$ and $F_o - F_c$ maps using the program O, version 6.2.2 (48). The quality of the electron density map was improved significantly by the program DM (45) with solvent flattening, histogram

matching and density modification options. At this refinement stage, non-crystallographic symmetry restraints were imposed on the four subunits in an asymmetric unit. NAD^+ molecules were incorporated into the model upon detection in F_o - F_c difference maps. The crystallographic refinement was further continued with the program CNS after the resolution range of refinement was extended to 40-1.7 Å. In the final refinement stages, the residues from 39 to 55 were excluded from NCS restraints in consideration of the conformational differences among the chains resulting from crystal packing. After the first round of refinement (R = 22.0%, R_{free} = 22.8%), water molecules having peak heights above 3.0 σ in the F_o - F_c maps and exhibiting acceptable hydrogen-bonding geometry were added to the model automatically using a water-pick option of CNS. Water molecules having B-values greater than 50 $Å^2$ were eliminated. The final R-value was 17.9% (R_{free} = 19.2%) for the resolution range of 40-1.7 Å. The stereochemistry of the model was verified using the software package PROCHECK (49). The refinement statistics are summarized in Table I.

RESULTS AND DISCUSSION

Quality of the Model

The crystal structure of GlcDH in complex with NAD^+ was determined by the molecular replacement method with chains A and C of 3α-HSDH as the search model. The model was refined to a crystallographic R-factor of 17.9% (R_{free} = 19.2%) for 94,821 unique reflections [$I_o > 2\sigma(I_o)$] in the resolution range of 40-1.7 Å. Table I summarizes the data collection and the results of the crystallographic refinement. A crystal asymmetric unit contains four chemically identical subunits (chains A, B, E, and F), four molecules of NAD^+, and 562 solvent molecules. The unit cell contains tetramers 1 and 2 (Fig. 1) comprising chains A, B, C, and D and chains E, F, G, and H, respectively. Tetramer 2 can be rotated around the R-axis by 84° into the same orientation as that of tetramer 1. The peptide chains were well defined in $2F_o$ - F_c maps. The pairwise rms discrepancies of the main chain atoms Cα, C, N, and O among the four crystallographically independent subunits vary at a value of 0.18 Å for pair A and B and 0.37 Å for pair A and E. The largest pairwise discrepancy along the chains was commonly observed at Lys41 located in the surface loop (Arg39-Asp43) between βB and αC, where secondary structure is described in the next section. Located at a contact region of crystal packing is Lys41 of chain A rendering a weak salt bridge with Asp108 of chain D in the neighboring tetramer. The loop region formed at residues Arg39 to Asp43 is flexible, as reflected in the higher B values by about 10 $Å^2$ than the average value of all residues, thereby making the conformation of the loop susceptible to crystal packing influence. Differences in conformation are mainly at residues Arg39 to Asp43 and at the N-terminal region of helix αC (Glu44-Lys53). The rms deviation among the four subunits of the main chain atoms excluding the segment from Arg39 to Lys53 was

0.039 Å. The conformation of these four crystallographically independent subunits is therefore essentially identical.

Subunit Structure

The subunit structure of GlcDH shares the typical fold of the short chain dehydrogenases/reductases of known structure. As shown in Fig. 3, the subunit of GlcDH folds into a single domain of the α/β doubly wound structure (51) consisting of seven-stranded central parallel β-sheet sandwiched by two arrays of three α-helices (αC; αB, and αG on one side and αD, αE, and αF on the other side). Secondary structure elements were assigned according to the convention described by Ghosh *et al.* (18) to allow easier comparison between GlcDH and the other SDR members of known structure. The two βαβαβ motifs, βA-αB-βB-αC-βC and βD-αE-βE-αF-βF, constitute the dinucleotide binding motif, the Rossmann fold (52). The motif βD-αE-βE-αF-βF-αG-βG, together with an adjoining loop from Asp255 to Gly261, is responsible for substrate binding and tetramer formation. A loop region from Asn192 to Ile218 including helices αFG1 and αFG2 in the present crystal structure is well defined in electron density maps. The loop region is stabilized by several hydrogen bonds between the protein and NAD^+ (see below), which are more numerous in GlcDH than in other SDRs except for *Datura stramonium* tropinone reductase II (TR-II) (36).

This loop region contributes to the binding of the substrate (19, 27-30, 33, 36). In apo-form or in complex with the cofactor, the loop region of SDR enzymes is disordered, whereas those in the ternary (enzyme-cofactor-substrate) complex are fixed because the substrate binding induces conformational change and stabilization of the loops (19, 27-30, 33). In the case of GlcDH, however, the loop region containing αFG1 and αFG2 is already fixed in the protein-NAD^+ complex where conformational change might have occurred upon NAD^+ binding, leading to stabilization of the loop in a conformation suitable for glucose binding. A similar phenomenon is also suggested for the structure of the ternary complex of TR-II (36).

The Conformation of NAD^+ and Its Binding Environment

Figure 2a shows the well-defined electron density of the NAD^+ molecule and the surrounding amino acid residues. The NAD^+ is bound to the enzyme in an extended conformation at the carboxyl ends of the central parallel β-strands. The overall conformation of the NAD^+ and its binding mode to the Rossmann fold is similar to those of the other SDRs, with the adenine ring in the *anti* conformation and the nicotinamide ring in the *syn* conformation (52). Both ribose rings have ^2E(C2'-*endo*) puckering. Nomenclature for the coenzyme atoms and sugar puckering forms is adopted from the polynucleotide IUPAC-IUB description (53). The distance between C6A of the adenine ring and C2N of the nicotinamide ring is 14.1 Å, a value very close to those in other SDRs (18, 21, 28, 31, 34). The NAD^+-protein interactions are

essentially identical among the four subunits in the asymmetric unit. There are also two well-ordered water molecules, which mediate hydrogen-bond interactions between NAD^+ and the interior surface of the protein. The interactions are shown in Fig. 2b and are summarized in Table II.

Nicotinamide Nucleoside Moiety

There are nine direct hydrogen bonds and one water-mediated hydrogen bond between the nicotinamide nucleoside moiety and the protein surface. The number of hydrogen bonds is greater in GlcDH than in other SDRs. Due to the close proximity of a loop region (residues 188-194) to the nicotinamide ring, additional interactions peculiar to GlcDH between this loop and the cofactor through hydrogen bonding may stabilize the loop region, which is essential for the formation of the active site cavity. A similar pattern of hydrogen bonding is found in TR-II (36). These interactions may allow the proper conformation of αFG1 and αFG2 for glucose binding.

Adenine Ring Moiety

No direct interactions are observed between the adenine ring and the internal surface of the protein. The adenine ring fits into a mostly hydrophobic pocket formed by Val66, Ala93 and the Cβ, Cγ, and Cδ atoms of Arg39. The plane of the Cβ, Cγ, and Cδ atoms is parallel to the adenine ring of NAD^+.

Adenine Ribose and Pyrophosphate Moiety

The adenine ribose and pyrophosphate moiety is situated on the turn between βA and αB (Gly14-Gly20), which is involved in the nucleotide-binding motif of the SDR members having the consensus sequence GXXXGXG (16). There are two hydrogen bonds between the protein and the adenine ribose and three hydrogen bonds between the protein and the pyrophosphate. Water-mediated hydrogen bonds are formed from the main chain Os of Gly14 and Thr17, the main chain NH of Gly20 and the OD1 of Asn92 to the O2'N and O5'PA of the NAD^+ as shown in Fig. 2b.

Coenzyme Specificity

GlcDH prefers $NADP^+$ to NAD^+ as the cofactor. The K_m value with $NADP^+$ as cofactor is about 10-fold lower than with NAD^+ (54). In most SDRs of known structure preferring NADP(H), two negative charges of the 2'-phosphate group are balanced by two positively charged residues (27, 29, 35, 36). One of the basic residues is the conserved arginine residue located at the turn following the βB strand. In GlcDH, Arg39 is close to O2PR of the adenine ribose. The distance of 3.75 Å between CD of Arg39 and C6A of the adenine of GlcDH is very similar to the value of 3.74 Å for mouse lung carbonyl reductase (MLCR) (29). The distance between NH2 of Arg39 and O2PR of the adenine ribose of GlcDH is, however, 1.37

Å longer than that of MLCR. If $NADP^+$ bound to GlcDH, the conformation of the side chain of Arg39 would change to compensate for the negative charge of 2'-phosphate group of $NADP^+$. The other positively charged residue is usually Lys or Arg, which is located in the fourth position of the GXXXGXG consensus sequence of the dinucleotide binding motif of the SDR family (29, 35, 36) except for human estrogenic 17β-hydroxysteroid dehydrogenase (17β-HSDH). A basic residue is lacking in the 4th position of the GXXXGXG of 17β-HSDH, but instead the charge was compensated for by the interaction between the 2'-phosphate and Lys195 located in the flexible loop following strand βF (27).

Thr17 of GlcDH located in the 4th position of the consensus sequence forms a hydrogen bond with the 2'-hydroxyl group of the adenine ribose. The well-defined electron density around the adenine ribose moiety strongly indicates that no basic residues other than Arg39 compensate for the negative charges of the 2'-phosphate group. In addition, there is no ambiguous electron density detected in the main chain of the flexible loop region between βF and αG, which would provide a second basic residue to the 2'-phosphate group. These findings suggest that in GlcDH, only one basic residue is enough to compensate for the negative charges of the 2'-phosphate group and to determine the coenzyme specificity.

The Active Site Architecture

The active site of GlcDH consisting of the nicotinamide moiety of NAD^+ and the highly conserved Ser-Tyr-Lys catalytic triad (16), namely, Ser145, Tyr158 and Lys162, is very similar to that of the other SDR enzymes. The conserved tyrosine residue (Tyr158) is considered as a general basic catalyst (29, 34, 36), and the pK_a value of its hydroxyl group is lowered by Lys162, leading to the stabilization of the tyrosinate anion at physiological pH (29, 34, 36).

The optimum pH of the oxidation reaction of GlcDH is 8.0 (12), whereas those of other SDR enzymes are greater than 8.5. The distance between Tyr158 OH and Lys162 NZ for GlcDH is 4.06 Å, the shortest among the SDRs of known structure. The distance between the two residues is 4.23 Å in 3α-HSDH, 4.28 Å in MLCR, 4.32 Å in *Pseudomonas* sp. *cis*-biphenyl-2,3-dihydrodiol-2,3-dehydrogenase (BphB), 4.32 Å in TR-II, 4.39 Å in *Drosophila lebanonensis* alcohol dehydrogenase (DADH), 4.54 Å in mouse sepiapterine reductase, 4.71 Å in 17β-HSDH, 4.84 Å in *Eschrichia coli* 7α-hydroxysteroid dehydrogenase (7α-HSDH) and 4.84 Å in *Mycobacterium tuberculosis* enoyl-acyl carrier protein reductase. It is likely that the shorter distance between Tyr158 OH and Lys162 NZ of GlcDH stabilizes the deprotonated form of Tyr158 at lower pH, thereby lowering its optimum pH.

Q-Axis Related Interface

The Q-axis related interface has the most extensive intersubunit interaction among the three axis related interfaces. The Q-axis interface of GlcDH comprises two long

helices αE and αF of subunit A, which form a four-helix bundle together with those of helices of subunit B. Hereafter, the word "subunit" is used instead of "chain." A second residue pair related by the 2-fold axis always accompanies a residue pair of interaction such as hydrogen bonding or hydrophobicity between one subunit and its symmetry mate. Hence here we describe only one of the two residue pairs in this section. Hydrophobic interactions especially of aromatic residues are predominant at the Q-axis interface of GlcDH (Fig. 4a) without forming any salt bridges. The Q-axis interface is subdivided into the αE-αE and αF-αF interfaces. In the αE-αE interface (Fig. 4a), the side chains of Trp109 in subunit A is stacked on that of Phe121 of subunit B. The hydrophobic residue Ile113 (A) faces Leu117 (B). Hydrogen bond interactions are listed in Table III.

Figure 5 shows the amino acid sequence alignment of the αE and αF helices among SDRs. In the αE-αE interface, four hydrophobic residues colored red are clustered in the space, of which interactions are partaken by two or more aromatic residues (Fig. 6). The αE-helix of GlcDH kinks at Leu117 (Fig. 4a) and similar kinks are also observed in other SDR enzymes (Fig. 6). In a dimeric SDR of DADH, the kink at αE serves to optimize the subunit-subunit interactions for dimer formation (33), while the kinks of 7α-HSDH, MLCR, BphB, and THNR serve to optimize the separation between the two αEs, in which every second and/or third hydrophobic residue is an aromatic residue. On the contrary, every second or third residue of GlcDH and 3α-HSDH is a small residue such as Ile, Leu, and Val, leading to less extensive hydrophobic interactions (Figs. 5 and 6). It is unlikely that the kinks of GlcDH and 3α-HSDH would contribute to the optimization of hydrophobic interactions at the regions.

The numbers of hydrogen bonds and salt bridges between the Q-axis related subunit pairs are summarized in Table IV. One pair of salt bridges further stabilizes the αE-αE interfaces of other SDRs (Fig. 6), whereas no salt bridge is observed in GlcDH. In addition, the total number of hydrogen bonds and salt bridges in GlcDH between the Q-axis related subunit pair is one of the smallest in the SDR family. The weakness of the αE-αE interface of GlcDH compared to other SDRs is attributable to less extensive hydrophobic interactions and the absence of ion pair interactions.

In contrast to the αE-αE interface, there are no large hydrophobic residues on the αF-αF interface. Instead, the small hydrophobic residues Val156, Ala159, Ala160, Leu167, and Thr171 of subunits A and B are found. Consequently, the two αF helices are situated closer to each other than those in the αE-αE interface. The amino acid sequence alignment of the αF-helices is shown in Fig. 5. In the case of GlcDH, the side chain of Met168, which does not face the inner side of the four-helical bundle, and Gly163 and Gly164 of GlcDH do not contribute to the hydrophobic interactions. There are also no direct hydrogen bonds at the αF-αF interface (Table IV). Thus, interactions at the αF-αF interface are also likely to be weaker in GlcDH than the other SDR enzymes.

P-Axis Related Interface

Interactions between the P-axis-related subunits are not extensive compared to those of the Q-axis. The P-axis interface consists of αG and βG of subunit A and those of symmetry-related subunit D (Fig. 4b). In the αG-αG interface, the indole ring of Trp230(A) is stacked on that of subinit D. No direct hydrogen bond interaction is found in the αG-αG and βG-βG interfaces, but hydrogen bonds are formed between other parts of the P-axis related subunits. Direct hydrogen bond interactions and water-mediated hydrogen bonds are summarized in Table III. The interactions through the P-interface of GlcDH are weaker than those through the Q-interface. The tetrameric SDRs have no conserved amino acid residue or characteristic sequence motifs at the αG-helix region, ever though they have similar folds. Table V compares the direct hydrogen bonds for the P-axis related subunits among the SDRs. There are no direct hydrogen bonds at the αG-αG interface, whereas about 20 or more direct hydrogen bonds and salt bridges are observed between the P-axis related subunits. The buried solvent-accessible area around the P-axis interface of GlcDH is 1,600 $Å^2$ per subunit, which is similar to the mean value of 1,680 $Å^2$ per subunit of the other SDR members. Accordingly, the P-axis related interface is of similar strength in GlcDH and the other SDRs. The weakness of P-interface must be a common feature of SDRs of known structure (18, 23, 28-30, 32).

R-Axis Related Interface

The R-axis interface contains only one prominent interaction: the side chain of Tyr253(A) is stacked on that of its symmetry-related subunit C (Fig. 4c). The C-terminal amino acid residues of subunit A [Phe255(A)-Gly261(A)] interact with a part of subunit C and are close to the active site cavity of subunit C. Hydrogen bonds are listed in Table III. GlcDH dissociates into inactive monomers at pH 9.0 (38-40). This suggests that the C-terminal region of subunit C is indispensable to the formation of the active site cavity of subunit A. Jany *et al.*, showed that GlcDH from *B. megaterium* M1286, which shows 82.8% sequence identity with GlcDH used in this study, is inactivated by chemical modification of Tyr253 (10, 15). The result indicated the importance of C-terminal region of R-axis related subunit C for formation of the active site cavity of subunit A.

In the tetrameric SDR enzymes, the four subunits are related by three mutually perpendicular 2-fold axes designated as P, Q, and R. The quaternary structures and the P- and Q-axis interfaces are similar among the tetrameric SDRs. Some SDRs have a few direct interactions among the R-axis related subunits, while others such as 7α-HSDH (28) and THNR (30) have none at all. Hence, the tetrameric structures of SDR enzymes, as well as that of GlcDH, are mainly maintained by the interactions through the P- and Q-axis interfaces. Only GlcDH exhibits a remarkable feature of a reversible dissociation-association of subunits under moderate conditions (38-40). Comparison of the subunit interactions reveals that the interactions between the P-axis related subunits are similar among SDR enzymes and the interactions of the P-interface are weak compared to the Q-interface.

Moreover, the interactions through the Q-axis related interface of GlcDH are weaker than those of other SDRs. The strength of the interactions of the Q-axis related interface, especially αE-αE contact, might be insufficient to maintain the tetrameric structure of GlcDH at pH 9.0.

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